Extracellular ATP in Activity-Dependent Remodeling of the Neuromuscular Junction

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ABSTRACT: **Electrical activity during early development affects the development and maintenance of synapses (Spitzer [2006]: Nature 4447:707–712), but the intercellular signals regulating maintenance of synapses are not well identified. At the neuromuscular junction, adenosine 5-triphosphate (ATP) is coreleased with acetylcholine at activated nerve terminals to modulate synaptic function. Here we use cocultured mouse motor neurons and muscle cells in a three-compartment cell culture chamber to test whether endogenously released ATP plays a role in activity-dependent maintenance of neuromuscular synapses. The results suggest that ATP release at the synapse counters the negative effect of electrical activity, thus stabilizing activated synapses. Confirming our previous work (Li et al. [2001]: Nat Neurosci 4:871–872), we found that in doubly innervated muscles, electrical stimulation induced heterosynaptic downregulation of the nonstimulated convergent input to the muscle fiber with no or little change of the stimulated inputs. However, in preparations that were stimulated in the presence of apyrase, an enzyme that degrades extracellular ATP, synapse downregulation of stimulated inputs was substantial and significant, and end plate potentials were reduced. Apyrase treatment for 20 h in the absence of stimulation did result in moderate diminution, but this was prevented by blocking spontaneous neural activity with tetrodotoxin. The P2 receptor blocker, suramin, also induced activity-dependent synapse diminution. The decrease in synaptic efficacy produced by prolonged stimulation in the presence of apyrase persisted for greater than 20 h, consistent with a developmental timecourse and distinct from the rapid neuromodulatory actions of ATP that have been demonstrated by others. We conclude that extracellular ATP promotes stabilization of the neuromuscular junction and may play a role in activity-dependent synaptic modification during development.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 924–932, 2007

Keywords: **synapse elimination; ATP; purinergic signaling; activity-dependent plasticity; neuromuscular junction; development**

INTRODUCTION

Early in development, muscle fibers are innervated by synaptic termininals from multiple axons. Eventually synaptic inputs from all but one axon to each muscle fiber are eliminated (Redfern, 1970). If impulse activity is blocked, synapse elimination does not proceed normally and junctions remain polyinnervated (Thompson et al., 1979). The molecular basis for this developmental synapse modulation is still being investigated (Jennings and Burden, 1993; Sadasvam et al., 2005). In previous studies using the *in vitro* chamber system described in the present work, we found that in muscle cells innervated by two synaptic inputs, electrical stimulation induced selective heterosynaptic decrease in efficacy and this was mediated in part by protein kinases A and C (Li et al., 2001, 2004; Nelson et al., 2003). The present experiments investigate the possible involvement of extracellular adenosine 5-triphosphate (ATP) in this process.

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ATP contained in synaptic vesicles is coreleased with neurotransmitter, where it has modulatory effects on synaptic transmission (Fu et al., 1997; Hong and Chang, 1998; Fields and Stevens-Graham, 2002; Fields and Burnstock, 2006; Todd and Robitaille, 2006). Direct application of ATP to cultured myotubes increased acetylcholine receptor (AChR) stability in the membrane (O'Malley et al., 1997), which is important for maintenance of neuromuscular junctions during synaptic remodeling in development (Balice-Gordon and Lichtman, 1994). Application of ATP induced expression of acetylcholine esterase and AChR in muscles (Tsim and Barnard, 2002), and differentiation of mammalian skeletal muscle (Ryten et al., 2002). These experiments do not, however, address the important question of whether the activity-dependent release of ATP at the NMJ stabilizes the synapse during the activity-dependent process of synapse elimination.

Terminal Schwann cells have also been implicated in synapse remodeling at the neuromuscular junction (Kang et al., 2003). Extracellular ATP modulates the strength of synaptic transmission at the NMJ by acting on terminal Schwann cells (Fields and Stevens-Graham, 2002; Auld and Robitaille, 2003; Todd et al., 2006). The present experiments were designed to test the hypothesis if extracellular ATP could contribute to activity-dependent synapse stabilization at the mouse NMJ, and if so, whether ATP can act independently from the known effects of ATP on perisynaptic Schwann cells. This was accomplished by using a coculture preparation of mouse motor neurons, astroglia, and muscle cells but no Schwann cells (see below). Additionally, this preparation enables experiments to determine the locus of activity-dependent ATP release.

Neuronal stimulation was applied acutely and for prolonged periods, and changes in synapse efficacy were measured in the presence and absence of the enzyme apyrase, which degrades extracellular ATP, or in the presence of the P2 receptor blocker, suramin. The results indicate that activity-dependent release of ATP stabilizes synapses at the NMJ, and ATP is released from both presynaptic and postsynaptic cells.

MATERIALS AND METHODS

Preparation

The tissue culture preparations involved three-compartment chambers with two populations of neurons from the ventral horn (VH) of the mouse spinal cord in combination with skeletal myotubes and were essentially as described in earlier work (Jia et al., 1999 for details of chamber fabrication and culture methodology). Neurons from the ventral half of the spinal cord were grown in the two-side chambers and skeletal muscle fibers in the center chamber. It should be noted that purified VH spinal cord neurons were grown on a feeder monolayer of cerebral cortical astrocytes. Staining of the VH/astrocyte cultures with three markers for Schwann cells (S100, the p75 neurotrophin receptor, and the specific Schwann cell marker—myelin protein zero or MPZ) indicated that there were no Schwann cells in these cultures. The neurites from the side chambers extending into the central chamber formed synapses with the muscle cells in the center chamber. The cultures were maintained in a 37°C, 10% CO_2 incubator.

Twitch Assay

A platinum electrode was placed in each side chamber and the ground electrode was in the center chamber. A stimulus delivered to axons from either side compartment elicited a contraction in some myotubes. This response was scored as a synaptic response. The percentage of functional synapse loss (FSL) was calculated as an index of the decrease of synaptic efficacy. The FSL was equal to the total number of initially responding myotubes minus the number of those myotubes responding after a treatment period, and this difference divided by the initial number of responses. See Jia et al. for details of this assay (Jia et al., 1999). In some experiments, chronic extracellular neuronal stimulation was performed in a CO_2 incubator. The stimulation pattern used in experiments was: 5 V biphasic (each pulse was 0.2 ms duration with a separation of 5 ms) electrical stimulation at a burst of 30 Hz, 2-s duration, every 10 s.

Intracellular Recording

Intracellular recordings were performed at room temperature in culture medium containing 5% horse serum. The pH was balanced with CO_2. Electrodes filled with 4 *M* potassium acetate solution had resistances of around 100 MΩ. Monosynaptic end plate potentials (EPPs) were evoked by extracellular stimulation of the axons from one side compartment. Data were collected through an interface (Instrutech), and analyzed on a G3 Mac computer with software developed by Synergy Research. The muscle sodium channel blocker, μ-conotoxin from BACHEM Bioscience (1 μM) was used to diminish the muscle action potential during intracellular recordings. In some cases, a variance analysis was done to determine synapse quantal variables. The mean quantal number or average number of quanta released per trial, *m*, is equal to 1/C.V. squared; the C.V. or coefficient of variation is equal to the standard deviation of the response amplitude divided by the response amplitude mean. The quantal amplitude is the mean response amplitude divided by *m*. For a more detailed description of this technique, see Li et al. (2002).

ATP Release Measurement

ATP concentration in the extracellular medium was measured using an ATP bioluminescence assay (Sigma Chemical Company), following the manufacturer's procedures. Experiments were conducted in full-culture medium, or in HEPES-buffered balanced salt solution, pH 7.3, at 35°C. Similar results were obtained in either medium, provided ample time (30 min) was provided after changing the culture medium. Ten microlitres of medium was collected at intervals of 0, 1, 3, and 5 min poststimulation, and added to 90 μL of reaction mix. Fluorescence intensity was measured immediately in a luminometer, and values were calibrated daily against a series of ATP standards. Electrical stimulation of 5 V, 0.2 ms pulses, at 10–30 Hz was applied in multicompartment chambers as described (Jia et al., 1999), or specific receptor agonists were added to the cultures by micropipette. In control experiments, measurements were taken after solution without agonist was added to the cultures, and the possible effect of agonists on the assay was checked by adding the drugs to ATP standard solutions. The latency to peak response varied among different preparations; therefore, the peak response within 5 min was used for data analysis in replicate experiments. Electrically-induced release of ATP from muscle cultures was also checked by direct observation on an inverted microscope equipped for single-photon imaging, as described (Cheng et al., 2001). This imaging method allowed direct observation of muscle cell contraction in response to electrical stimulation in glass-bottomed culture dishes, through field electrodes, simultaneously with ATP measurements. Because of the lower impedance between electrodes in these cell cultures, higher voltage stimulation was applied (10–25 V). The glia cultures were the same cerebral cortical astrocytes used as a feeder layer in the VH-cultures.

Chemicals

Apyrase (200 units) from Sigma Chemical, for the experiments was dissolved in culture medium to a final concentration of 30 unit/mL. Suramin from Sigma Chemical was dissolved in H_2O to make a 10 m*M* stock solution. This was diluted 100-fold with culture medium to make a final concentration of 100 μM for application to the cells. ATP was from Sigma Chemical. Tetrodotoxin (TTx) from Sigma Chemical (1 μM) was used to block spontaneous activities. Veratridine was dissolved in ethanol to make a 10 m*M* stock and used at 50–100 μM. Carbachol was dissolved in PBS and used at 0.1–1 m*M*.

Results are presented as the mean ± the standard error of the mean.

RESULTS

ATP Is Released From Both Nerve and Muscle by Activation

Sources of activity-dependent release of ATP at the neuromuscular junction were identified by measuring ATP with a luciferin/luciferase reaction in various cultures stimulated by depolarization with veratridine (100 μM), with 10 Hz electrical stimulation, or by AChR activation with carbachol (1 m*M*). An over 15-fold increase of ATP was produced in neuronal cultures by the addition of veratridine. Since these neurons are cultured on a background of cortical astrocytes, we tested responses in pure cultures of astrocytes to the same veratridine treatment, but no increase in ATP was measurable. This argues against astrocytes as the predominant source of ATP in these neuronal cultures.

An increase (>3-fold) in ATP release from neuronal cultures was produced by the electrical stimulation. Carbachol produced an over 17-fold increase in ATP release from muscle cultures and produced a marginal (<2-fold) increase in ATP release from astrocytes. These astrocytes are not present in muscle cultures and therefore can not be the source for the large increase in ATP release induced by carbachol from muscle cultures. As an example of this sort of experiment, ATP release from myotubes in culture in response to electrical stimulation is shown in Figure 1. Figure 1(A) shows digital images of the dots of light associated with individual molecules of ATP, and Figure 1(B) plots counts of the concentration of ATP in the culture medium as a function of time during electrical stimulation for 2 s at 30 Hz every 10 s.

Apyrase Treatment Causes Activity-Dependent Synapse Reduction

We wished to explore the effect of apyrase on synaptic efficacy and have used the twitch assay of synaptic strength to this end (Fig. 2). In these experiments, only stimulated inputs were examined, and as we have noted, stimulated inputs are stable in untreated preparations. Two hours of stimulation in the presence of apyrase gives highly significant synapse loss (loss 21.7%, $p < 0.004$ compared with stim only) as measured by the twitch assay, and there is significantly less loss in the presence of apyrase but with no stimulation (loss 8.8%, $p < 0.021$ relative to stim. plus apyrase). When the treatment/stimulation period was increased to 20 h, the loss with apyrase treatment, both in the presence (loss 27.4%, $p = 0.017$ compared with stim only) and absence of electrical stimulation (loss 29.6%, $p = 0.001$ compared with no stim, no apyrase control group) was increased. The loss with apyrase in the absence of stimulation was presumably related to spontaneous activity in the cultures, since when activity was blocked with TTx in the presence of apyrase, there was no significant

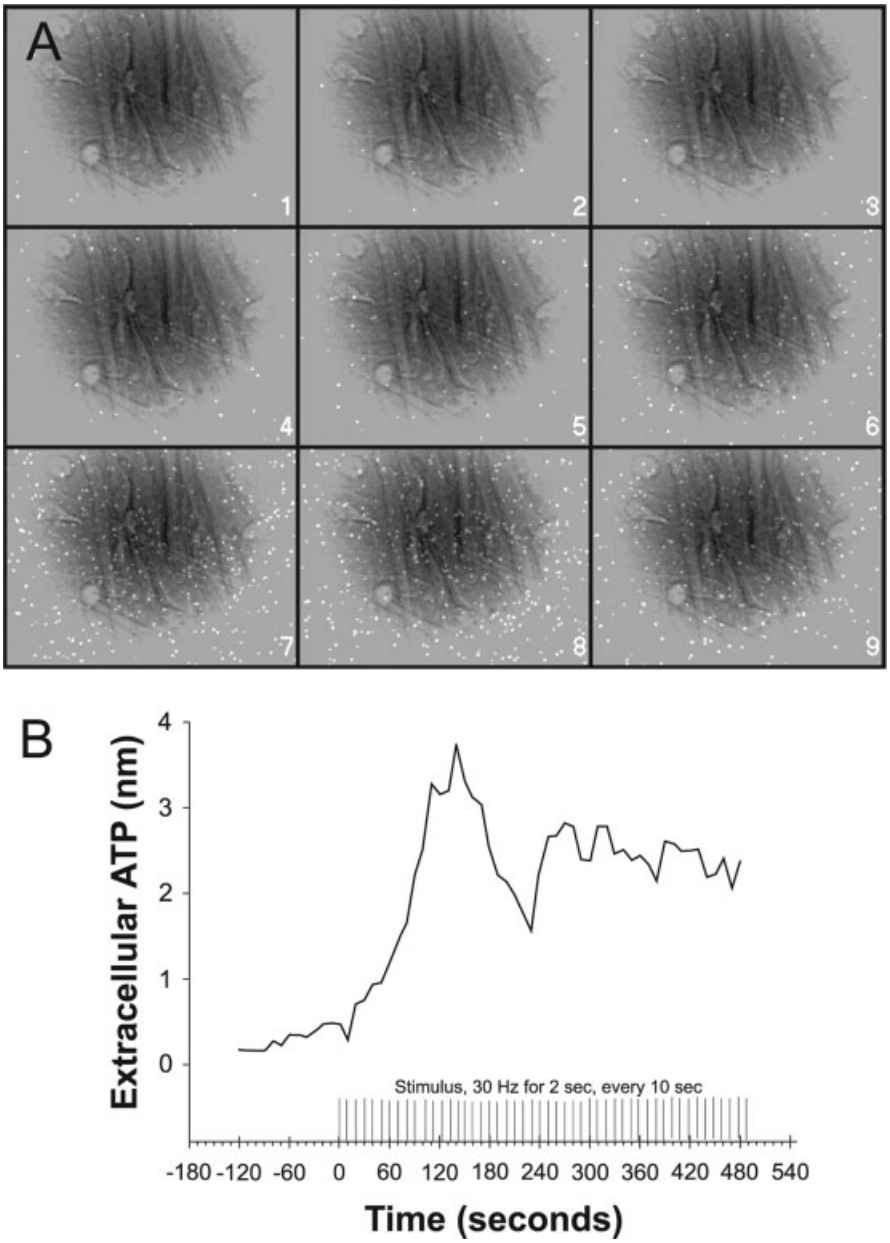

Figure 1 Imaging of ATP release in a muscle culture with electrical stimulation for 2 s at 30 Hz every 10 s using single photon imaging in the presence of luciferin/luciferase. White dots represent light emissions from individual ATP molecules in (A), with each frame taken sequentially. (B) Counts of the emissions as a function of time after electrical stimulation, converted to nanomolar ATP using a calibration curve derived from known concentrations of ATP.

loss of synaptic connectivity (loss 7.2%, $p > 0.8$ compared with control), i.e., the loss in apyrase and TTx was the same as with TTx and no apyrase ($p > 0.3$). In our system, treatment with TTx alone had no effect on synapse efficacy as measured with the twitch assay (data not shown).

As noted, previous results had shown that when neural inputs to the muscle cells in our system are stimulated, these stimulated inputs maintain their synaptic efficacy quite well, with relatively little decrement produced by activation. When similar stimulation was done in the presence of the enzyme, apyrase, which breaks down ATP, however, we measured a considerable loss of synaptic efficacy. This is shown in Figure 3, in which intracellular recordings from innervated myotubes are taken as an

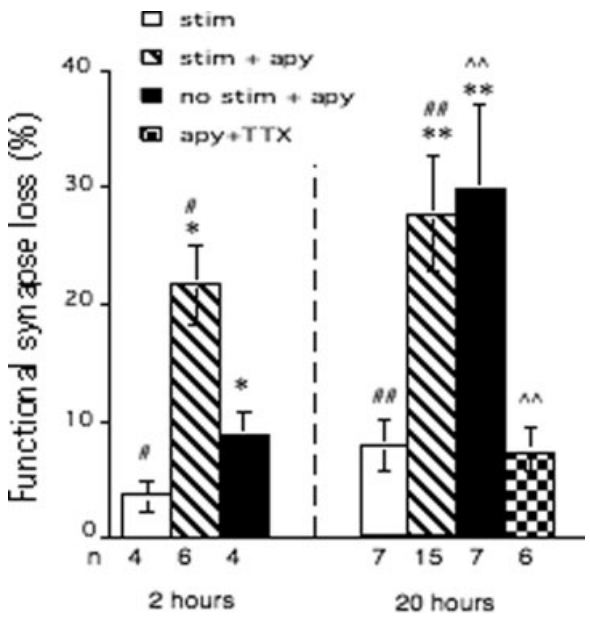

Figure 2 Application of 30 units/mL of apyrase induced significant synapse downregulation in electrically stimulated preparations at both 2 and 20 h. #$p < 0.004$ and ##$p < 0.018$. The synapse decrement is more in 2 h of apyrase treatment with electrical stimulation than in spontaneously active but unstimulated preparations treated with apyrase (*$p < 0.024$). In preparations treated with apyrase for 20 h there was no significant difference (**$p > 0.8$) between stimulated preparations and spontaneously active but unstimulated preparations. Preparations treated with both apyrase and TTx (to block all electrical activity) had significantly less loss than either of these preparations and were no different from untreated control preparations. In this and subsequent figures the error bars represent the SEM.

indicator of synapse strength. Averaged synaptic responses are shown before and after stimulation for 2 h in the presence of apyrase. Amplitude histograms are shown in the second row of the figure. The shift to lower amplitude responses after stimulation is clear. The bottom graph shows data from five different synaptic connections that were stimulated in the absence of apyrase and which showed no decrement in strength with the stimulation (left pair of bars). The pair of bars on the right represent data from eight different synaptic connections that were stimulated the same as in the other part of the figure, but in the presence of apyrase. The decrement in synapse strength is to less than 50% of the prestimulus value, a highly significant loss. In these experiments, the apyrase was added to both the center and side compartments to ensure that no dilution of the apyrase solution by leakage of medium would take place. Similar decrements in synapse efficacy with stimulation were obtained when agents were added only to the center, synaptic compartment (see below).

Synapse Stabilization Is Mediated by Activation of P2 Receptors

ATP activates P2-type purinoceptors, either ionotropic (P_2X) or G-protein coupled (P_2Y), and it can also be hydrolyzed by ectonucleotidases to adenosine, which activates P1 receptors. To test whether ATP exerts its effects on P2 receptors at the neuromuscular junction, a P2 receptor blocker, suramin, was used. Similar to apyrase, suramin also induced activity-

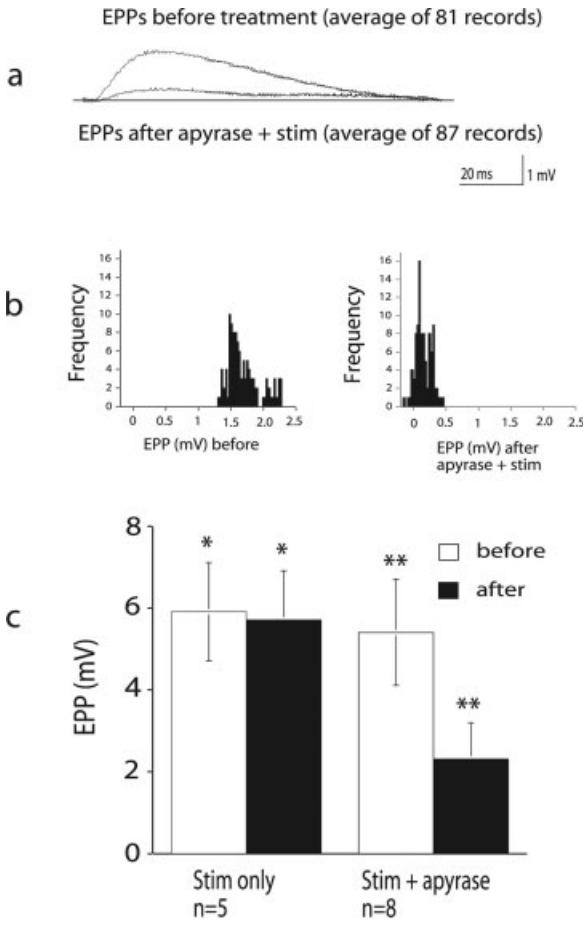

Figure 3 Intracellular recordings show that a treatment of apyrase (2 h) reduced EPP amplitude. (a) EPP traces before (upper) and after (lower) the treatment of apyrase plus electrical stimulation. (b) The histogram of the same recordings in (a). (c) EPPs from five control (electric stimulation only) and eight experiments (electrical stimulation plus apyrase) showed that the 2-h treatment of apyrase plus electrical stimulation reduced EPP amplitude (*control group: $p > 0.47$, **apy + stim group: $p < 0.027$; paired t test).

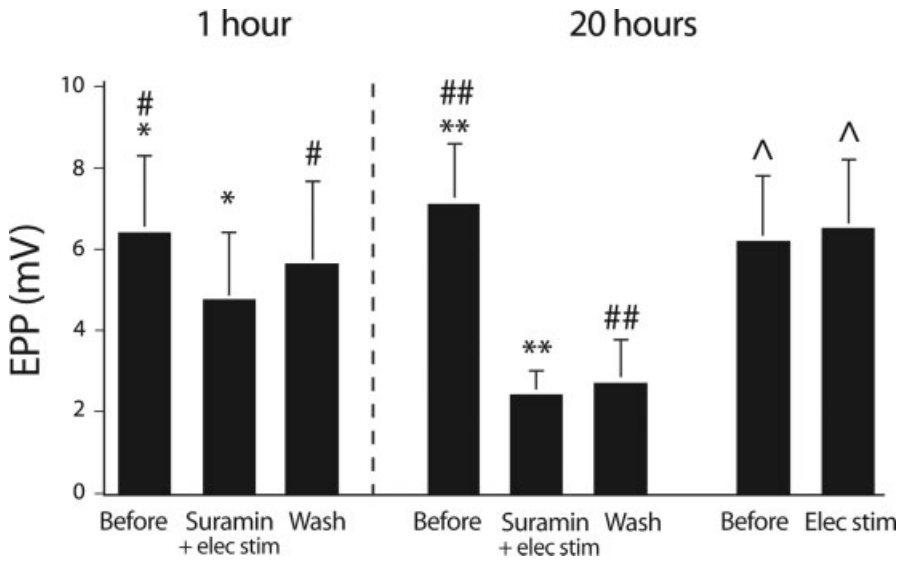

Figure 4 ATP activates P2-type purinoceptors. The P2 receptor blocker, suramin, also induced activity-dependent synapse reduction. Suramin (100 μM) added in the center chamber (synapse part) induced EPP reduction with either 1 h ($n = 5$, $*p < 0.018$) or 20 h ($n = 6$, $**p < 0.019$) electrical stimulation. EPP amplitudes recovered after wash out of suramin following the 1 h treatment of suramin plus stimulation ($n = 5$, $\#p > 0.2$). EPPs did not recover after wash out suramin following 20 h treatment with suramin plus electrical stimulation ($n = 6$, $\#\#p < 0.015$). In a control group with electrical stimulation for 20 h in the absence of suramin did not induce EPP reduction ($n = 4$, $\wedge\, p > 0.4$; paired t test).

dependent synapse diminution in our preparations. With suramin (100 μM) added only in the center synaptic chamber and using the twitch assay, electrical stimulation induced synapse diminution after either 2 h (ca. 28% loss) or 20 h (47% loss) (data not shown). EPP recordings show that suramin only in the center chamber plus stimulation reduced EPP amplitude (Fig. 4). After a 20-h treatment, EPP amplitude was significantly reduced ($p < 0.019$). After wash out of suramin, the average EPP amplitude did not recover immediately ($p < 0.015$, compared with control). These results suggest that the effects of extracellular ATP on synapse stabilization are through the activation of P2 receptors. The effects are not readily reversed by washout of the suramin. Also note that even after 20 h of stimulation in the absence of ATP receptor blockade there was no reduction in EPP amplitude (two right hand bars).

ATP Acts Presynaptically

We have used a variance analysis method to determine whether the reduction in synaptic efficacy was due to a decrease in transmitter release probability or quantal number (a presynaptic variable) or to a decrease in quantal amplitude (a postsynaptic variable). We have previously shown that this analysis is an appropriate one in our preparation (Li et al., 2002). In four synapses stimulated for 2 h in the presence of apyrase in side and center chambers and in which the recordings were stable and there was at least a 2-fold change in EPP amplitude, the results were clear. There was a decrease in the quantal number, m, to 16% of the prestimulation value (36.4 $\pm$ 11.7 to 5.8 $\pm$ 2.2 mean $\pm$ S.E.M; $p = 0.041$). There was a nonsignificant change in quantal amplitude (0.139 $\pm$ 0.09 to 0.08 $\pm$ 0.02; $p > 0.2$). This result was confirmed in experiments in which apyrase was added only to the central, synapse compartment of the chambers and the preparations were stimulated for about 1 h. In four connections there was a significant decrease in quantal number ($p = 0.02$) with no change in quantal size ($p > 0.3$).

ATP Has Long Term Effects (>20 h)

We were interested in the possibility that ATP might be involved in relatively long-term modulation of synaptic function. We have examined the recovery from 20-h treatments with apyrase. The recovery process is shown in Figure 5. After some 20 h of the apyrase/stimulation regimen, recovery from the pro-

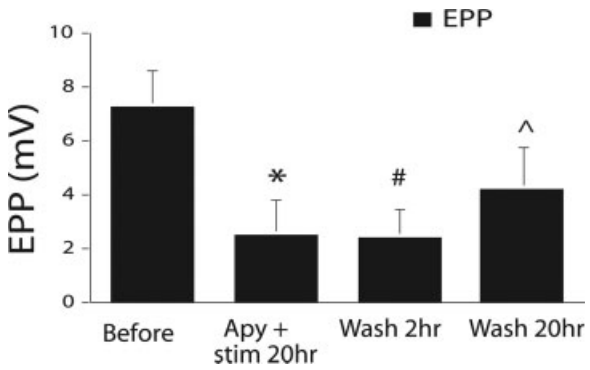

Figure 5 A treatment of apyrase plus electrical stimulation for 20 h reduced the EPP ($n = 8$, $*p < 0.014$). Two hours and 20 h after the wash out of apyrase and stopping electrical stimulation the EPP reductions are still significant ($\#p < 0.02$ and $\wedge\, p < 0.039$; paired t test).

longed treatment is slow, and even 20 h after washout of the apyrase and cessation of stimulation, significant synaptic decrement could be demonstrated (Fig. 5). We conclude that in the absence of extracellular ATP, activity produces some relatively long-term changes in synaptic function, or stated conversely, ATP plays a positive role in maintaining synaptic function during activation.

Exogenous ATP Does Not Stabilize Synapse

Some discrepancy exists in the literature as to the effects of exogenously applied ATP on synaptic function (Fu et al., 1997; Giniatullin and Sokolova, 1998). We have examined this question in our chamber system, using several concentrations of ATP, from 0.1 to 100 μM, applied in the center, side, or both compartments of the chamber system. ATP at a concentration of 100 μM applied in the center chamber produced a synapse loss of (72 ± 5)%, $n = 6$, as measured by the twitch assay, a highly significant loss. Using the EPP assay, 100 μM ATP application induced a decrease from 7.3 ± 1.6 to 1.1 ± mV, $n = 6$. Thus, the EPP was reduced to 18% of the pretreatment value. For 10 μM ATP the value was 27%, $n = 5$; for 1 μM 53%, $n = 3$ and for 0.1 μM there was no reduction, $n = 3$.

Using the twitch assay we found a significant difference when ATP was applied in the different compartments of our system. As noted above, 100 μM ATP in only the center chamber produced a (72 ± 5)% loss of functional connections. Significantly less loss, (25 ± 6)%, $n = 3$, was produced when the ATP was applied in both the center and side compartments. No loss was produced (0 ± 0)% when the ATP was applied only in the side compartment containing the VH neurons. We have not studied this further but it suggests some positive effect on synaptic transmission of ATP applied to the neuronal cell bodies.

DISCUSSION

A major problem for neuroscience is to determine the mechanisms for activity-dependent Hebbian synaptic plasticity, particularly during development (Fields and Nelson, 1992; Nelson et al., 1995). Our findings show that ATP is released in an activity dependent manner from both nerve and muscle, and we suggest that the released ATP could play a role in developmental synapse modulation. In our system, activation of a neuromuscular synapse results in minimal or no loss of synaptic efficacy of that activated synapse. There is a destabilizing effect of activation, however, since an inactive input to the activated muscle is diminished in efficacy. If extracellular ATP action is blocked by the application of apyrase (which breaks down extracellular ATP), stimulation produces a highly significant (>50%) reduction in synapse strength of the stimulated input. We interpret this as indicating that ATP, coreleased with Ach by neural activity, serves to stabilize and maintain efficacy of stimulated synapses.

The ATP P2 receptor appeared to be involved in the preservation of synaptic strength during chronic stimulation, since preparations incubated in the P2 blocker, suramin, exhibited substantial synaptic loss when stimulated. Our previous work has shown that PKA activation also stabilizes synapses (Nelson et al., 2003). It seems likely that ATP works on synapses at least in part through a process parallel to the PKA cascade.

The effects of ATP breakdown by apyrase required neuromuscular activity to be evident; in the presence of the Na+ channel blocker, TTx, apyrase had no effect on synapse efficacy. Thus, lack of extracellular ATP does not have a direct negative effect on synaptic transmission. ATP release appears to counter the negative effect of electrical activity, thus stabilizing activated synapses. Local ATP levels in the absence of activity in a given synapse were not adequate to maintain that synapse, in that inactive synapses were reduced by activation of the muscle cells by other inputs. It is important to note that both of the molecules eliciting activity-dependent synapse loss can act only on extracellular (released) ATP. Each acts by a different mechanism, with apyrase reducing extracellular ATP and suramin blocking P2 ATP receptors.

Both nerve and muscle cells responded to activation with an increase in ATP release, so either nerve or muscle (or both) cell types could be responsible for the ATP-related effects on synapse strength. Our quantal analysis indicated that the long-term (>2 h) effects of ATP were due to a reduction in the presynaptically determined quantal number (mean number of released neurotransmitter quanta). The quantal size was not significantly affected. Thus, whatever the source of the released ATP, one of the targets for its action is the presynaptic nerve. However, ATP can act through several different receptors and has been shown to affect AChR stability (O'Malley et al., 1997). It seems a plausible possibility that ATP may act also, in part, by preventing loss of the postsynaptic receptor from activated synapses. Schwann cells have been shown to modulate transmission through multiple mechanisms (Fields and Stevens-Graham, 2002; Auld and Robitaille, 2003). However, we failed to detect staining in our cultures with three separate Schwann cell markers and conclude that Schwann cells are absent. Our results, therefore show an effect of activity-dependently released ATP on synaptic function that is not dependent on these perisynaptic Schwann cells.

There is a striking difference between the effects of ATP released by nerve stimulation and ATP generally applied in the bathing medium. Since block or breakdown of ATP produces an activity-dependent decrease of synapse effectiveness, we conclude that endogenously released ATP stabilizes synapses. We found, by contrast, that exogenously applied ATP over a range of concentrations from 100 to 1 μM reduces synapse strength. This may reflect differences in the temporal or spatial properties of receptor activation by endogenous and exogenously applied ATP. Moreover, stimulation of extrasynaptic purinergic receptors by exogenous ATP could have different effects from activation of synaptic receptors by ATP released at the presynaptic terminal. It may be noted that suppression of ACh release, mediated by P2 receptors, has been demonstrated at the NMJ (Giniatullin and Sokolova, 1998), but at a concentration of 1 mM ATP, (Fu et al., 1997), found an increase in Ach release. Our finding of differential effects of ATP applied to the center or side compartments of our chamber system may be relevant to these different results. It may be noted that all P2 receptors, with the possible exception of P2X6, P2X7, P2Y1, and P2Y11 would be expected to be activated by ATP at a concentration of 100 μM (Burnstock and Knight, 2004).

The loss of synapse efficacy produced by block of activity-dependent synaptically released ATP suggests that this locally generated extracellular ATP acts to strengthen or stabilize synaptic function. Our observations deal with physiological, functional indices of synaptic efficacy, and it will be important to examine structural features that may be related to ATP action such as alteration of postsynaptic receptors or presynaptic boutons.

Our previous results (Li et al., 2001, 2004) showed an involvement of protein kinases A and C in selective activity-dependent synapse reduction and stabilization. In particular, PKC activation served to destabilize synapses and PKA stabilized them. Those mechanisms presumably work in concert with the ATP initiated effects noted here.

The effects of interfering for prolonged periods of time (2–20 h) with extracellular ATP action were long lasting (at least 20 h). This suggests to us that ATP action may be involved in long-term modulation of synapse structure and function and since production of this ATP is activity dependent, the ATP may be involved in the activity-dependent modulation of synaptic circuitry that occurs during development.

REFERENCES

Auld DS, Robitaille R. 2003. Perisynaptic Schwann cells at the neuromuscular junction: Nerve- and activity-dependent contributions to synaptic efficacy, plasticity, and reinnervation. Neuroscientist 9:144–157.

Balice-Gordon RJ, Lichtman JW. 1994. Long-term synapse loss induced by focal blockade of postsynaptic receptors. Nature 372:519–524.

Burnstock G, Knight GE. 2004. Cellular distribution and functions of P2 receptor subtypes in defferent systems. Int Rev Cytol 240:31–304.

Cheng P, Lin B, Kao F, Gu M, Xu M, Gan X, Huang M, et al. 2001. Multi-photon fluorescence microscopy—The response of plant cells to high intensity illumination. Micron 32:661–669.

Fields RD, Burnstock G. 2006. Purinergic signaling in neuron-glia interactions. Nat Rev Neurosci 7:423–436.

Fields RD, Nelson PG. 1992. Activity-dependent development of the vertebrate nervous system. Int Rev Neurobiol 34:133–214.

Fields RD, Stevens-Graham B. 2002. New insights into neuron-glia communication. Science 298:556–562.

Fu WM, Chen YH, Lee KF, Liou JC. 1997. Regulation of quantal transmitter secretion by ATP and protein kinases at developing neuromuscular synapses. Eur J Neurosci 9: 676–685.

Giniatullin RA, Sokolova EM. 1998. ATP and adenosine inhibit transmitter release at the frog neuromuscular junction through distinct presynaptic receptors. Br J Pharmacol 124:839–844.

Hong SJ, Chang CC. 1998. Evaluation of intrinsic modulation of synaptic transmission by ATP in mouse fast twitch muscle. J Neurophysiol 80:2550–2558.

Jennings CG, Burden SJ. 1993. Development of the neuromuscular synapse. Curr Opin Neurobiol 3:75–81.

Jia M, Li M, Dunlap V, Nelson PG. 1999. The thrombin receptor mediates functional activity-dependent neuromuscular synapse reduction via protein kinase C activation in vitro. J Neurobiol 38:369–381.

Kang H, Tian L, Thompson W. 2003. Terminal Schwann cells guide the reinnervation of muscle after nerve injury. J Neurocytol 32:975–985.

Li MX, Jia M, Jiang H, Dunlap V, Nelson PG. 2001. Opposing actions of protein kinase A and C mediate Hebbian synaptic plasticity. Nat Neurosci 4:871–872.

Li MX, Jia M, Yang LX, Dunlap V, Nelson PG. 2002. Pre- and postsynaptic mechanisms in Hebbian activity-dependent synapse modification. J Neurobiol 52:241–250.

Li MX, Jia M, Yang LX, Jiang H, Lanuza MA, Gonzalez CM, Nelson PG. 2004. The role of the theta isoform of protein kinase C (PKC) in activity-dependent synapse elimination: Evidence from the PKC theta knock-out mouse in vivo and in vitro. J Neurosci 24:3762–3769.

Nelson PG, Fields RD, Liu Y. 1995. Neural activity, neuron-glia relationships, and synapse development. Perspect Dev Neurobiol 2:399–407.

Nelson PG, Jia M, Li MX. 2003. Protein kinases and Hebbian function. Neuroscientist 9:110–116.

O'Malley JP, Moore CT, Salpeter MM. 1997. Stabilization of acetylcholine receptors by exogenous ATP and its reversal by cAMP and calcium. J Cell Biol 138:159–165.

Redfern PA. 1970. Neuromuscular transmission in new-born rats. J Physiol 209:701–709.

Ryten M, Dunn PM, Neary JT, Burnstock G. 2002. ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X5 receptor on satellite cells. J Cell Biol 158:345–355.

Sadasvam G, Willmann R, Lin S, Erb-Vogtil S, Kong XC, Ruegg MA, Fuhrer C. 2005. Src-family kinases stabilize the neuromuscular synapse in vivo via protein interactions, phosphorylation, and cytoskeletal linkage of acetylcholine receptors. J Neuroscience 25:10479–10493.

Spitzer NE. 2006. Electrical activity in early neuronal development. Nature 4447:707–712.

Thompson W, Kuffler DP, Jansen JK. 1979. The effect of prolonged, reversible block of nerve impulses on the elimination of polyneuronal innervation of new-born rat skeletal muscle fibers. Neuroscience 4:271–281.

Todd KJ, Robitaille R. 2006. Purinergic modulation of synaptic signalling at the neuromuscular junction. Pflugers Arch 452:608–614.

Todd KJ, Serrano A, Lacaille JC, Robitaille R. 2006. Glial cells in synaptic plasticity. J Physiol Paris 99:75–83.

Tsim KW, Barnard EA. 2002. The signaling pathways mediated by P2Y nucleotide receptors in the formation and maintenance of the skeletal neuromuscular junction. Neurosignals 11:58–64.